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14. ABSTRACT- Bone is a highly vascularized tissue reliant on the close spatial and temporal connection between blood vessels and bone cells to maintain skeletal integrity. Several experimental studies have shown that angiogenesis plays a vital role in skeletal development and bone fracture repair. 1) Studies using a distraction osteogenesis model (DO) have shown that intramembranous bone formation is induced by the application of gradual mechanical distraction across an osteotomy defect, which reveals not only an increase in osteogenesis but also an increase in expression of several angiogenic factors (1). 2) Treadmill-running in rats displayed bone marrow angiogenesis concomitant with increase in osteogenesis (2). 3) Studies using mandibular DO model have shown that high frequency traction provides a proper mechanical environment for angiogenesis contributing to enhanced bone formation (3). These findings illustrate that angiogenesis, osteogenesis and ML are tightly related. Recently, newly discovered MicroRNA (miR)'s belonging to a small class of RNA molecules have received considerable attention because of the ability to act as a negative regulator of gene expression. So far, at least 500 MicroRNAs have been discovered of which few are linked to pathogenesis of disease (4-7). In particular, MicroRNA92a has been reported to control angiogenesis. Since angiogenesis and osteogenesis are critical for increasing bone healing and bone strength in the rehabilitation program, we predict that inhibiting miR that control angiogenesis can maximize the benefits of exercise on skeleton. Based on this and the above rationale, we propose the hypothesis that blocking miR that control angiogenesis will increase the magnitude of anabolic effects of ML on bone formation. To test this, two specific aims are proposed.					
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INTRODUCTION

Bone is a highly vascularized tissue reliant on the close spatial and temporal connection between blood vessels and bone cells to maintain skeletal integrity. Several experimental studies have shown that angiogenesis plays a vital role in skeletal development and bone fracture repair. 1) Studies using a distraction osteogenesis model (DO) have shown that intramembranous bone formation is induced by the application of gradual mechanical distraction across an osteotomy defect, which reveals not only an increase in osteogenesis but also an increase in expression of several angiogenic factors (1). 2) Treadmill-running in rats displayed bone marrow angiogenesis concomitant with increase in osteogenesis (2). 3) Studies using mandibular DO model have shown that high frequency traction provides a proper mechanical environment for angiogenesis contributing to enhanced bone formation (3). These findings illustrate that angiogenesis, osteogenesis and ML are tightly related. Recently, newly discovered MicroRNA (miR)'s belonging to a small class of RNA molecules have received considerable attention because of the ability to act as a negative regulator of gene expression. So far, at least 500 MicroRNAs have been discovered of which few are linked to pathogenesis of disease (4-7). In particular, MicroRNA92a has been reported to control angiogenesis. Since angiogenesis and osteogenesis are critical for increasing bone healing and bone strength in the rehabilitation program, we predict that inhibiting miR that control angiogenesis can maximize the benefits of exercise on skeleton. Based on this and the above rationale, we propose the hypothesis that blocking miR that control angiogenesis will increase the magnitude of anabolic effects of ML on bone formation. To test this, two specific aims are proposed.

Specific aim 1: Assess bone anabolic response to ML, and then, evaluate expression levels of ms that are known to control angiogenesis and identify those that are involved in bone response to ML.

- 1) Subject 10 week C57BL/6J mice to tibia axial loading.
- 2) Evaluate expression levels of MicroRNA that are known to control angiogenesis by using Real time RT-PCR approach after 6-hours, 24- hours, 3- & 7-days of axial loading.
- 3) Evaluate if there is an association between bone formation markers, angiogenesis markers and miR levels by performing correlation analysis.

Specific aim 2: Determine the consequence of blocking identified miR using antisense oligos complementary to the specific miR that control angiogenesis on loading induced bone formation.

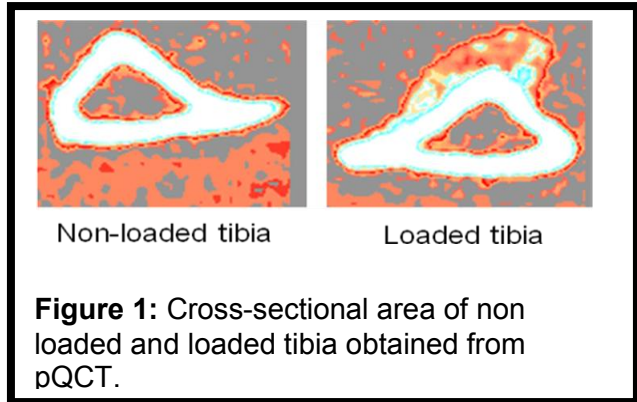
- 1) Using an *in vitro* model, determine the efficiency of antagomirs specific for identified miR that control angiogenesis.
- 2) Based on *in vitro* results and specific aim 1, we will inject mice with antisense oligos (antagomirs) specific for miR that control angiogenesis.
- 3) Subject this experimental group and control mice to axial loading for 2 weeks.
- 4) Measure ML induced changes in skeletal parameters and bone strength by Micro-CT.
- 5) Measure cellular processes contributing to osteogenesis and angiogenesis by performing histology.

BODY

(Progress report for the period 1 - 18 months)

The proposed grant involves use of animal testing to evaluate the above specific aims. Therefore, during first 4 months of the grant proposal, animal protocols were written to obtain approval from the IACUC committee. Once the approval was obtained, 9 week old female C57BL/6J (B6) mice were ordered from Jackson Laboratory to evaluate the first specific aim. After 1 week of housing, at 10 weeks of age, a single dose of 10N load was applied on tibia of these mice and the **study was terminated at different time points (6-hours, 24- hours, 3- & 7-days of axial loading)**. Mice were euthanized using CO₂ and tibias (loaded and non-loaded bones) were collected, marrow flushed and used for RNA extraction. Quality and quantity of RNA were analyzed using 2100 Bio-analyzer (Agilent, Palo Alto, CA, USA) and Nano-drop (Wilmington, DE) and the RNA was stored at -80°C for further experiments.

Earlier studies, using animal models have reported a list of miR's that are known to regulate angiogenesis. Among these, for this study, we selected only the miR that show high expression levels and has a high inhibitory effect on angiogenesis. Therefore, we purchased Taqman miR kits from Applied Biosystem. Taqman miR assay involves a simple two step protocol that requires reverse transcription with a mRNA-specific primer followed by real time PCR with Taqman probes. Using 10ng of RNA, we evaluated the expression levels of selected miR in RNA samples isolated from externally loaded and non-loaded bones. Furthermore, we also evaluated the expression levels of bone formation and angiogenic marker genes in the same RNA samples. **The results from our study revealed no change either in bone formation markers, miR or angiogenic genes. A potential explanation for not seeing any change in the expression levels of genes is that the duration of loading may not be adequate to stimulate the biological response.** Therefore, we further, extended our study by performing a longer duration of loading using the same loading model. Nevertheless, to assure that this is not due to the in vivo loading model, we also selected another method of loading, four-point bending. The rationale for selecting this loading model was based on earlier reports (8).



Selection of in vivo loading model that stimulates angiogenesis coupled osteogenesis

Four-point bending method of loading: We purchased two sets of B6 mice from Jackson laboratory. In one set of mice, at 10 week age, a 9N load was applied on the right tibia of B6 mice using a **four-point bending device** (Instron, CA) while the left tibia was used as internal control. The loading was performed at 2Hz frequency, for 36 cycles, once per day for 2 weeks (6 days/ weeks and 1 day rest). 48 hours, after the last day of loading, in vivo pQCT [Stratec Medizintechnik, Berlin, Germany] was performed on the diaphysis area (loading region) of tibia to determine if there was any significant change in the geometrical properties of loaded tibia when compared to non-loaded tibia. Routine calibration was performed daily with a defined standard containing hydroxyapatite crystals embedded in Lucite. Scanning was performed using the manufacturer supplied software program, designed to analyze the data and generate the values for the change in bone parameters. Furthermore, the X-ray attenuation data were analyzed based upon the software-defined threshold. We set up two thresholds for our analysis. A 180-730 mg/cm³ threshold was used to measure total area and bone mineral content (BMC) in the loaded vs. unloaded bones and a 730-730-mg/cm³ threshold was used to measure total volumetric density (vBMD). The rationale for selecting this threshold is to include the newly formed mineralized bone. In order to minimize the measurement errors caused by positioning of tibia for pQCT, we used the tibia-fibular junction as the reference line. We selected four-slices that start 3 mm proximal from tibia-fibular junction for pQCT measurement. This region corresponds to the loading zone. Each slice is at a 1mm interval and the values presented in the results are an average of these four slices (8).

As can be seen in Figure 1, in

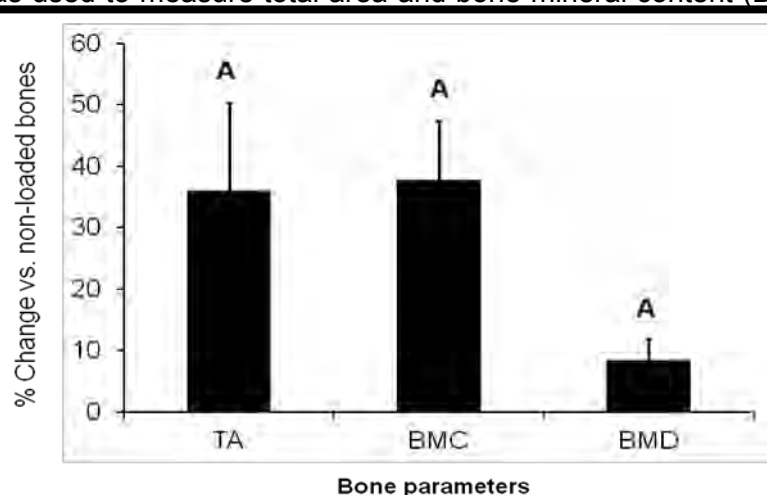
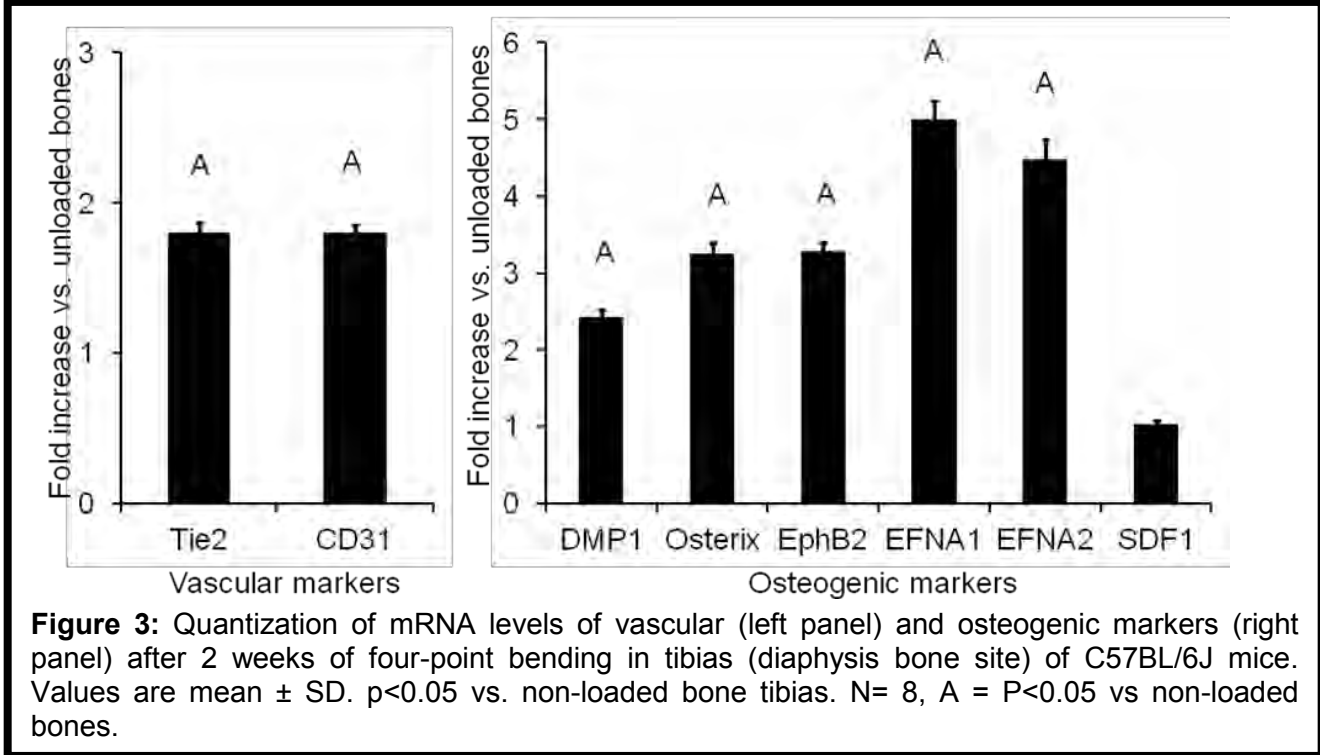


Figure 2: Percent change in bone parameters in response to 2 weeks of four-point bending measured by pQCT. Values are mean ± SD. p<0.05 vs. non-loaded bone tibias. TA-Total area, BMC-bone mineral content, BMD, total volumetric bone density, N= 8. A = P<0.05 vs. non-loaded bones.

response to 2 weeks of four-point bending, the loaded bones of B6 mice show significant increase in the newly formed bone when compared to non-loaded bones. Furthermore, quantization of skeletal parameters, shown in figure 2, revealed 8 to 30% increase in total area, bone mineral content and total volumetric density.

Subsequently, we euthanized the mice and collected tibias (loaded and non-loaded bones) and stored at -80°C for further experiments. RNA was extracted from these bones using our previously published protocol (8). Quality and quantity of RNA were analyzed using a 2100 Bio-analyzer (Agilent,



Palo Alto, CA, USA) and Nano-drop (Wilmington, DE). Gene expression analysis was performed on these RNA samples using Real time RT-PCR approach. Briefly, purified total RNA [200 μ g/ μ l] was used to synthesize the first strand cDNA by reverse transcription according to the manufacturer's instructions [Bio-Rad, CA]. The gene specific primers for bone formation (DMP1, osterix, EphB2, EFNA1, EFNA2, SDF1) and angiogenesis (CD 31, Tie 2) were designed by using Vector NTI software and ordered from IDT DNA technologies. Approximately 25 μ l of reaction volume was used for the real time PCR assay that consisted of 1X [12.5 μ l] Universal SYBR green PCR master mix [Master mix consists of SYBR Green dye, reaction buffers, dNTPs mix, and Hot Start Taq polymerase] [Applied Biosystems, Foster City, CA], 50nM of primers, 24 μ l of water, and 1 μ l of template. The thermal conditions consisted of an initial denaturation at 95°C for 10 minutes followed by 40 cycles of denaturation at 95°C for 15 seconds (sec), annealing and extension at 60°C for 1 minute, and a final step melting curve of 95°C for 15 sec, 60°C for 15 sec, and 95°C for 15 sec. All reactions were carried out in duplicate to reduce variation. The data were analyzed using SDS software, version 2.0, and the results were exported to Microsoft Excel for further analysis. Data normalization was accomplished using the endogenous control [β -actin] and the normalized values were subjected to a $2^{-\Delta\Delta C_t}$ formula to calculate the fold

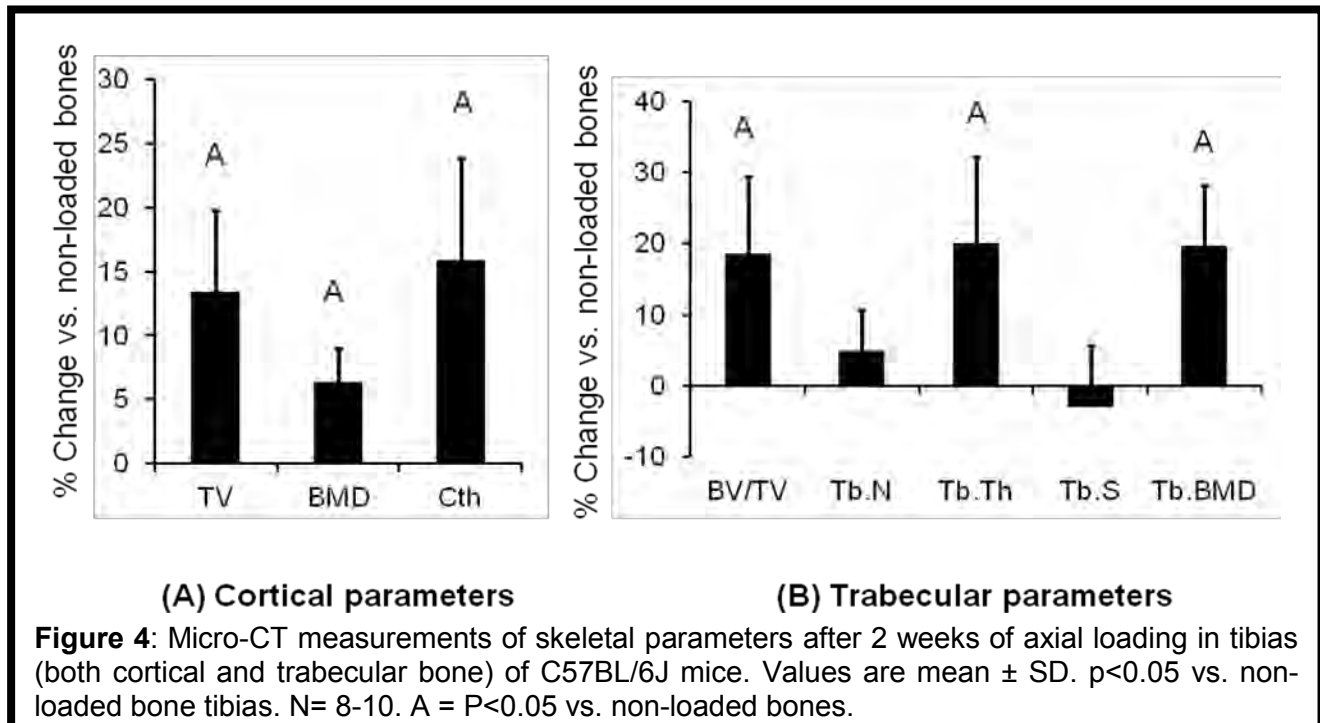
Bone genes	Angiogenic markers	
	CD31	Tie-2
DMP1	0.60 (p=0.04)	0.21
Osterix	0.52 (p=0.07)	0.55 (p=0.06)
EFNB1	-0.39	-0.40
EFNA2	-0.25	0.65 (p=0.04)
EphB2	0.56 (p=0.06)	0.57 (p=0.06)

Table-1: Correlation analysis between bone marker genes vs. angiogenic marker genes after 2 weeks of four-point bending. N=8-9.

change between the loaded and non-loaded tibias. The rationale for selecting the above bone marker genes and angiogenic genes was based on our previously published microarray findings and past reports (9, 10). As can be seen in Figure 3, in response to 2 weeks of four-point bending, the loaded bones of B6 mice show significant change in the expression levels of both osteogenic and angiogenic genes when compared to non-loaded bones. Furthermore, to assure that the increase in bone anabolic response is in part due to increase in vascular markers, we performed correlation analysis. Accordingly, vascular markers CD 31 and Tie-2 show significant as well as positive correlation with osteogenic markers (Table-1). Together, these data are consistent with the possibility that osteogenesis induced by four-point bending is in part mediated by an increase in angiogenesis.

Axial method of loading: In another set of B6 mice, at 10 weeks of age, a 10N axial load (Trapezoidal-shaped pulse period=0.1s [loading 0.025s, hold 0.05s and unloading 0.025s]; rest time between pulse=10 s; cycles/day=40) was applied on the right tibia and the left tibia was used as non-loaded internal control. The loading was performed at 3 alternate days/ week for 2 weeks. 48 hours after the last day of loading, in vivo micro-CT was performed on the loaded and non-loaded bone to evaluate loading induced changes in skeletal parameters. The rationale for choosing micro-CT measurement for this loading model is that this model induces both cortical and trabecular bone response and pQCT cannot measure trabecular bone response to loading as efficiently as micro-CT.

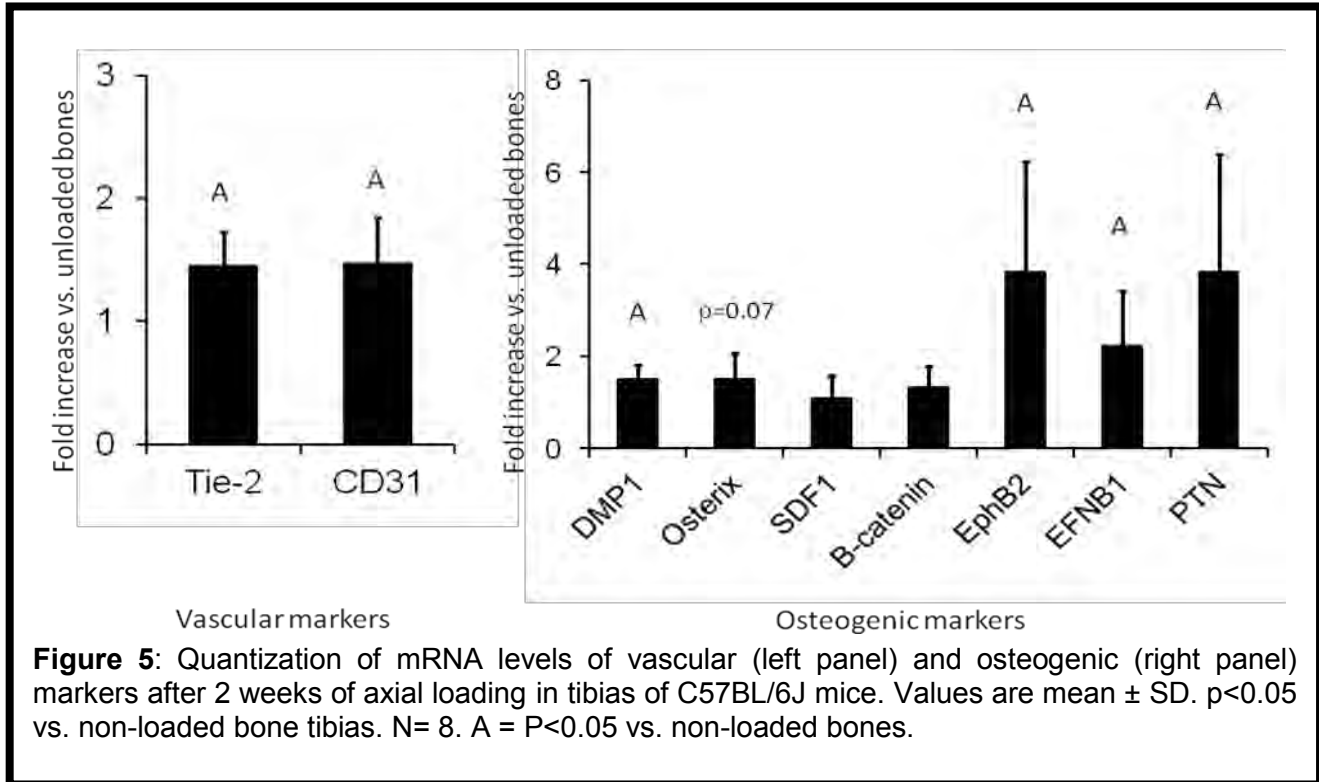
To measure microarchitectural changes of trabecular bone as well as cortical bone in response to axial loading, we used micro-CT, a high resolution tomography image system (Scanco Invivo CT40, Switzerland). Routine calibration was performed once per week using a three-point calibration phantom corresponding to the density range from air to cortical bone. Bones were immersed in 1X PBS to prevent them from drying and scanning was performed using 75Kv X-ray at a resolution of 10.5 μ m. To minimize the position error (slice positioning) and to be consistent in our sampling site from mouse to mouse, we undertook several precautionary steps, which included: 1) the use of scout view of the whole tibia to



determine landmarks and precise selections of measurement sites; 2) the use of the growth plate of the tibia as the reference point; 3) use of a 0.525 mm sampling site that represented 0.315 mm away from the growth plate for measurement of trabecular bone parameters and 4) the use of a 1.05 mm sampling site that represented 5.5 mm away from growth plate for measurement of cortical bone parameters. After acquiring the radiographic data, images were reconstructed by using 2-D image software provided by Scanco. The area of the trabecular analysis was outlined within the trabecular compartment. Every 10 sections were outlined, and the intermediate sections were interpolated with the contouring algorithm to

create a volume of interest, followed by three dimensional analysis using Scanco in vivo software. Parameters such as bone volume (BV, mm³), bone volume fraction (BV/TV, %), apparent density (mg HA/ccm), trabecular number (Tb.N, mm⁻¹), trabecular thickness (Tb.Th, μ m) and trabecular space (Tb.Sp, μ m) were evaluated in the externally loaded right and non-loaded left tibia of B6 mice.

In response to 2 weeks of axial loading, the loaded bones of B6 mice revealed 7 to 13% increase in the cortical bone parameters (total volume, bone density and cortical thickness) as shown in Figure 4. While at the trabecular site, the loaded bones show 18-20% increase in bone volume/tissue volume, thickness followed by density with decrease in space.



Subsequently, we also euthanized the same mice and collected tibias (loaded and non-loaded bones) and stored at -80°C for further experiment. RNA was extracted from these bones using our previously published protocol (9). Quality and quantity of RNA were analyzed using a 2100 Bio-analyzer (Agilent, Palo Alto, CA, USA) and Nano-drop (Wilmington, DE). Gene expression analysis was performed on these samples using Real time RT-PCR approach as described above for the four-point bending RNA samples. As shown in Figure 5, the gene expression analysis revealed significant increase in the expression levels of both osteogenic and vascular genes in response to axial loading. Furthermore, correlation analysis revealed that there is an association between angiogenesis marker genes and osteogenic marker genes. This finding indicates that axial loading induced increase in bone anabolic response is in part mediated by an increase in angiogenesis.

Though both four point bending and axial loading model induces osteogenic coupled vascular effect in response to loading, the magnitude of increase was higher in the four-point bending method of loading than the axial

Bone genes	Angiogenic markers	
	CD31	Tie-2
Osterix	0.40	0.84 ($p=0.03$)
EpHB2	-0.04	0.16
EFNA2	-0.41	-0.29
EFNB1	0.88 ($p=0.04$)	0.20
DMP1	0.79 ($p=0.05$)	-0.19
PTN	0.58	-0.19

Table-2: Correlation analysis between bone marker genes vs. angiogenic marker genes after 2 weeks of axial loading, N=6.

method of loading. Therefore, we chose four-point bending method of loading for further study.

Using the above loading model and regimen, we then, performed mechanical loading by four-point bending method on tibia of 10 week old female B6 mice for 2 weeks. After the last day of loading, RNA was extracted followed by Real time RT-PCR was performed on these sample to evaluate the expression levels of 7 selected miR (mir-93, mir15b, mir-20a, mir-146a, mir-16, mir-221, mir-222 and internal control U6) that are known to regulate angiogenesis.

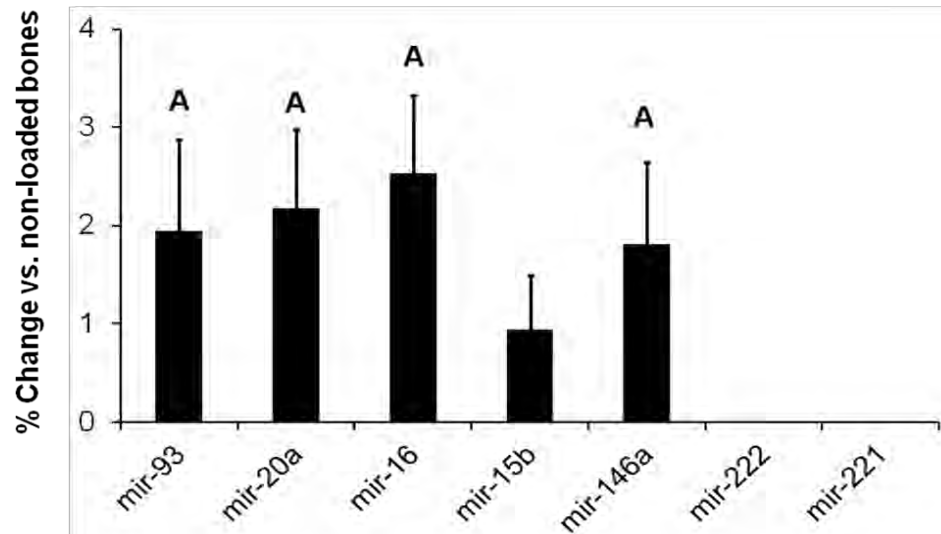


Figure 6: Quantization of miR levels after 2 weeks of four-point bending in tibias (diaphysis bone site) of C57BL/6J mice. A=P<0.05 vs. non-loaded bones (One-Way ANOVA), N=7.

As can be seen in Figure 6, among these miR's, one way ANOVA analysis revealed that the loaded bones of B6 mice showed significant increase (2-fold, $p < 0.05$ vs. non-loaded bones) in miR-93, miR-20a and mir-16, and miR-146a when compared to non-loaded bones while mir-15b showed no change in response to loading. miR-221 and-222 levels were below detectable levels using Taqman Assay kits. Since these miR's have been shown to be expressed in other cell types, it is possible that that the expression of miR-221 and miR-222 may be cell type specific (11). Correlation analyses revealed a positive correlation between miR-20a and the two angiogenic markers and several osteogenic markers studied. In contrast, miR-93 showed a positive correlation with Tie-2 but not CD31 and negative correlation with several osteogenic molecules while miR-16 showed a positive correlation with CD31 and osteogenic molecules and a negative correlation with Tie-2. miR-15b showed a negative correlation with

Osteogenic genes	MicroRNA					
		miR-93	miR-20a	miR-16	miR-15b	miR-146a
	EFNB1	-0.00	0.02	0.04	-0.13	0.23
	Osterix	-0.05	0.75	0.44	0.16	0.84
	DMP1	-0.54	0.39	0.51	-0.21	0.34
	EphB2	-0.58	0.85	0.48	0.01	0.28
	ENFA2	0.30	-0.18	0.05	-0.71	-0.01
Angiogenic genes	CD31	-0.29	0.60	0.62	-0.51	0.37
	Tie-2	0.63	0.52	-0.53	-0.12	0.12

Table-3: Correlation analysis between expression levels of miR, bone and vascular genes after 2 weeks of four-point bending. N=7

CD31, Tie-2 and ENFA2. miR-146a showed positive correlation between several osteogenic molecules

and CD31. We also evaluated miR-92 expression, which have been reported as a major negative regulator of angiogenesis. However, the probe did work and we are waiting for a replacement from the Applied Biosystem. Since individual miR's have their own mRNA targets, it is not surprising that the correlation between various osteogenic and angiogenic markers is not consistent for a given microRNA. In any case, our data suggest that some miR's may promote angiogenesis while others act as inhibitors.

In addition, we also performed axial method of loading on B6 mice using the above protocol, extracted RNA and evaluated expression levels of miR-93,-20a, 16 and 15b. The results from our study revealed no change in any of the above miR's in the loaded bones vs. non-loaded bones in response to axial method of loading. This finding provides additional evidence for selecting four-point bending method of loading for this study and to show that the magnitude of bone response induced by loading is critical to see a change in miR expression.

(Progress report for the final year of the funding)

During the first year of the funding, we identified miR that is associated with angiogenesis and osteogenesis. Instead to selecting the above microRNA for further in vitro testing and validation, we **focused on the miR-92 because a recent study demonstrated that over expression of miR92 blocked 80% angiogenesis in human endothelial cells and that blocking miR92 recovered mouse**

from limb ischemia injury as well as from myocardial infarction, respectively (2).

Since angiogenesis and osteogenesis tightly coupled, we predicted that inhibiting miR92 that control angiogenesis can maximize the benefits of exercise on skeleton. Based on this and the above rationale, we propose the hypothesis that blocking MicroRNA that control angiogenesis will increase the magnitude of anabolic effects of ML on bone formation.

Table - 4: Body weight and tissue weight measurements in mice treated with antagomir (experimental group) vs. control antagomir (control group) after two weeks of four-point bending in 10 week old female B6 mice.

Tissues	Control group (grams)	Experimental group (grams)
Body weight	19.6 \pm 0.1	19.7 \pm 0.26
Kidney	0.10 \pm 0.006	0.098 \pm 0.005
Liver	0.88 \pm 0.02	0.83 \pm 0.04
Heart	0.10 \pm 0.006	0.11 \pm 0.010

N=4, Values are mean \pm SD.

Animals: To test the above prediction, 9 week old female C57BL/6J mice were purchased from Jackson Laboratory. All the mice were housed under the standard conditions of 14-hour light and 10-hour darkness, and had free access to food and water. The experimental protocols were in compliance with animal welfare regulations and approved by local IACUC.

Antagomir designing and dosage: To block miR-92, a microRNA that is well known in regulating angiogenesis, single stranded RNA antagomir sequences against miR-92 and control antagomir sequence were ordered from IDT DNA technology. The sequences were obtained from a previously published manuscript (2). In the miR-92 antagomir and control antagomir, the 2'O RNA base was methylated followed by first two bases and last 3 bases were phosphorothiated to increase the stability of antagomir from degradation. In addition, a cholesterol-TEG was added at the 3' for easy entry of antagomir into the cells. The sequence of antagomir for miR-92a is as follows: 5'-CAGGCCGGGACAAGUGCAAUA-3') and Antagomir-Co (5'- AAGGCAAGCUGACCCUGAAGUU-3'.

Several studies have used antagomir to block target microRNA at a concentration ranging from 0.33 mg to 100 mg/kg of bodyweight in animal models. The issue of whether the doses used are optimal and specific to target microRNA has not been examined thoroughly. However, one potential concern with the use of high dose of antagomir is that antagomir at high concentrations could produce non-specific effects by inhibiting other genes besides target genes. Since reports have shown 0.33- 1.0- and 3.3- mg/kg of dosage was effective in exhibiting the biological response in tissues (12), we chose a dose of 2.7mg/kg of bodyweight antagomir for our study.

Four-point bending in antagomir treated mice and micro-CT measurement of skeletal parameters: To test, if blocking miR-92 maximizes the benefit of exercise on bone, we performed ML using four-point bending method on 10 week old female B6 mice for a period of 2 weeks (8-10). We choose this model and the load based on our findings that showed high osteogenic and angiogenic effect in response to loading during the first year of funding (8). We applied a 9N load on the right tibia of B6 mice over the muscle and soft tissue at 2Hz frequency, 36 cycles once per day under inhalable anesthesia (5% Isoflurane and 95% oxygen) for a period of 2 weeks (6 days/week with 1 day rest). The left tibia was used as contra-lateral internal control. During the loading regimen we injected mice with antagomir against miR92 and control antagomir via retro-orbital approach under 5% Isoflurane and 95% oxygen anesthesia for a period of one week (3 alternate days).

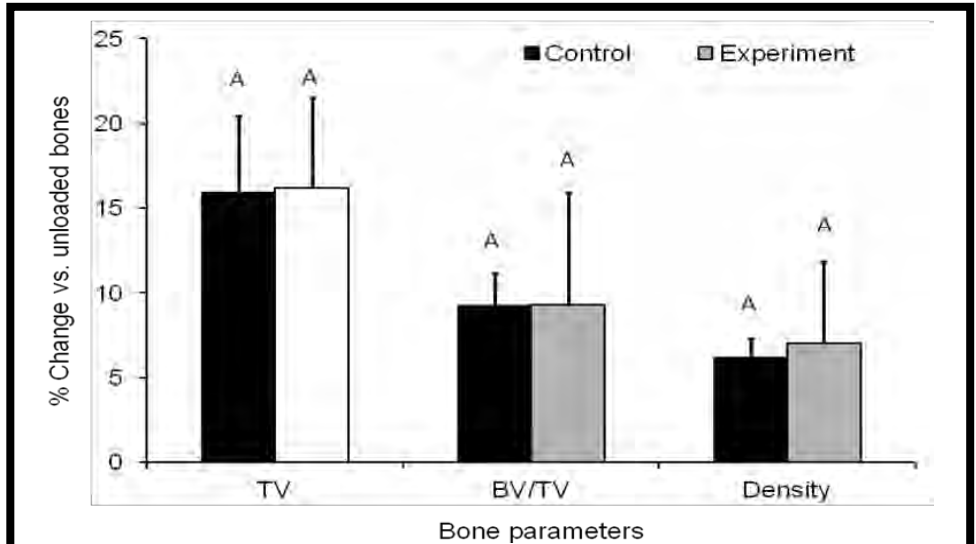


Figure 7 – Micro-CT measurements of bone parameters (diaphysis area) after 2 weeks of loading in female B6 mice treated with control antagomir and antagomir against miR-92. The y-axis corresponds to percent change and x-axis represents bone parameters. TV: Tissue volume, BV/TV: Bone volume/Tissue volume and density: total bone density. Values are mean \pm SD, N=4. ^Ap<0.05 vs. unloaded bones.

After two weeks of loading, we measured mice body weight and tissues weights after euthanization followed by tibias were collected to evaluate if there is an increase or decrease in loading induced changes in skeletal parameters by using micro-CT (Scanco Invivo CT40, Switzerland) and histology (9).

After two weeks of loading, we found no significant differences in the body weight between control and experiment group (Table-4).

Similarly, we did not see a difference in the tissue weight between the control and experimental groups suggesting that there are no side effects caused by the antagomir injection (Table-4). Micro-CT analysis revealed that two weeks of ML increased tibia tissue volume (TV) and bone volume/tissue volume (BV/TV) and bone density by 15%, as expected, in the control antagomir treated mice. Surprisingly, similar increases in TV (16%), BV/TV (9%) and bone density (7%) were also seen in mice treated with miR-92 antagomir (Figure 7). Since the antagomir used did not sensitize the skeleton to mechanical loading, as we predicted, we did not perform any histomorphometric analysis on the bone samples from this study.

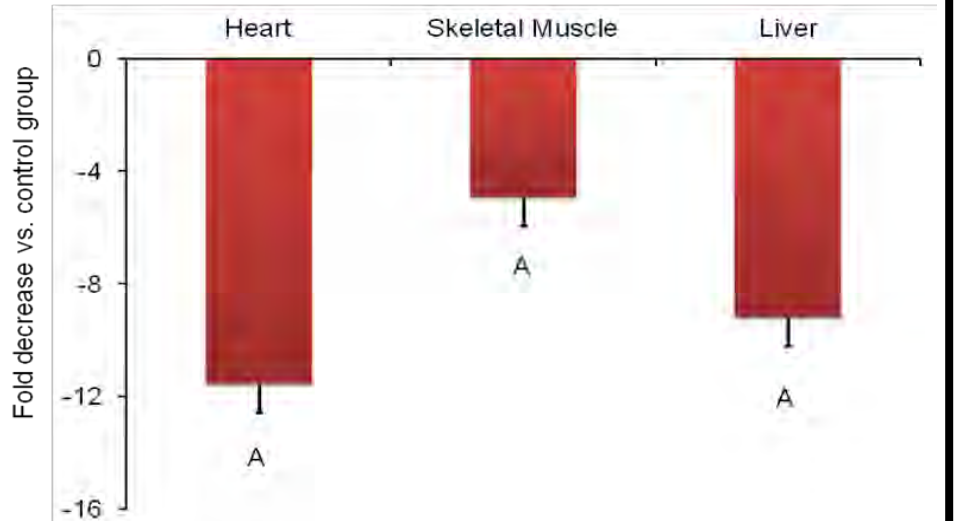


Figure 8 – Expression levels of mir-92 in heart, skeletal muscle and liver from mice treated with antagomir against mir-92 vs. control antagomir treated mice. The y-axis corresponds to fold change and x-axis represents different tissues. Values are mean \pm SD, N=4. ^Ap<0.05 vs. mice treated with control antagomir.

Since there was no difference between the groups, this, then raises a question whether systemic administration of mir-92 antagonist was effective in reducing the levels of miR-92. To test this, RNA was extracted from liver, heart, and skeletal muscle of antagonist and control antagonist treated mice using Trizol followed by Direct-zol kit (Zymo Research, USA). Quality and quantity of RNA were analyzed using a 2100 Bio-analyzer (Agilent, Palo Alto, CA, USA) and Nano-drop (Wilmington, DE). To assure that the antagonist delivered into mouse model blocked miR-92, expression levels of miR-92, U6 (internal control) and two other MicroRNA's (miR-93 and miR-20a) to determine the specificity were quantitated in liver, skeletal muscle and heart of mir-92 antagonist and control antagonist treated mice. Levels of MicroRNA's were evaluated using microRNA specific stem-loop RT primer and by Real time PCR primer that were obtained from Applied Biosystem and reactions were performed according to the manufacture instructions. In brief, 10ng of RNA was used to synthesize the first strand of cDNA by reverse transcription using respective stem-loop microRNA RT primer. Approximately 10 μ l of reaction volume was used for the RT assay that consisted of 0.1 μ l of 100 dNTPs, 1 μ l of 10X Buffer, 0.5 μ l of reverse transcriptase enzyme, 0.12 μ l of 50U/ μ l RNasin, 1 μ l of 5X RT primer, 6.78 μ l of Nuclease free water and 0.5 μ l of 10ng RNA. 5 μ l of the five times diluted first strand cDNA reaction was subjected to real time PCR amplification using microRNA specific real time PCR primers. Approximately 20 μ l of reaction volume was used for the assay that consisted of 10 μ l of TaqMan 2X Universal PCR Master mix, no AmpErase UNG, 0.5 μ l of 20X probe against target microRNA and 4.5 μ l of Nuclease free water, and 5 μ l of DNA. The data were analyzed using SDS software, version 2.0, and the results were exported to Microsoft Excel for further analysis. Data normalization was accomplished using U6 as internal control and the normalized values were subjected to a $2^{-\Delta\Delta C_t}$ formula to calculate the fold change between the antagonist and control antagonist treated groups. The results from our study revealed a 5-14 fold decrease in miR-92 levels in the heart, liver and skeletal muscles of mice treated with miR-92 antagonist compared to control antagonist (**Figure 8**). In contrast, expression levels of two other MicroRNA's miR-93 (fold change vs. control antagonist = 1.0 ± 0.6) and -20a (0.88 ± 0.18) were not different between the two groups of mice, thus suggesting specificity of the antagonist used. Since past reports have shown that mir-92 is an important regulator of angiogenesis, we also determined whether blocking miR-92 maximized the expression levels of vascular marker genes in the above tissues samples. We used CD31, Tie2 and VEGF genes to evaluate angiogenesis since reports have shown that these two genes are highly expressed on the surface of the endothelial cells at sites of neo-vascularization or vascularization and an increase in the expression levels of these genes corresponds to an increase in vascularization (13-15). To quantitate expression levels of vascular genes, briefly, purified total RNA [200 μ g/ μ l] was used to synthesize the first strand cDNA by reverse transcription according to the manufacturer's instructions [Bio-Rad, Hercules, CA USA]. 5 μ l of the five times diluted first strand cDNA reaction, was subjected to real time PCR amplification using gene specific primers as described earlier (10). The gene specific primers for angiogenesis (CD 31, Tie 2) were designed by using Vector NTI software and ordered from IDT DNA technologies (USA). Approximately 20 μ l reaction volume was used for the real time PCR assay that consisted of 10 μ l Universal SYBR green PCR master mix [Master mix consists of SYBR Green dye, reaction buffers, dNTPs mix, and

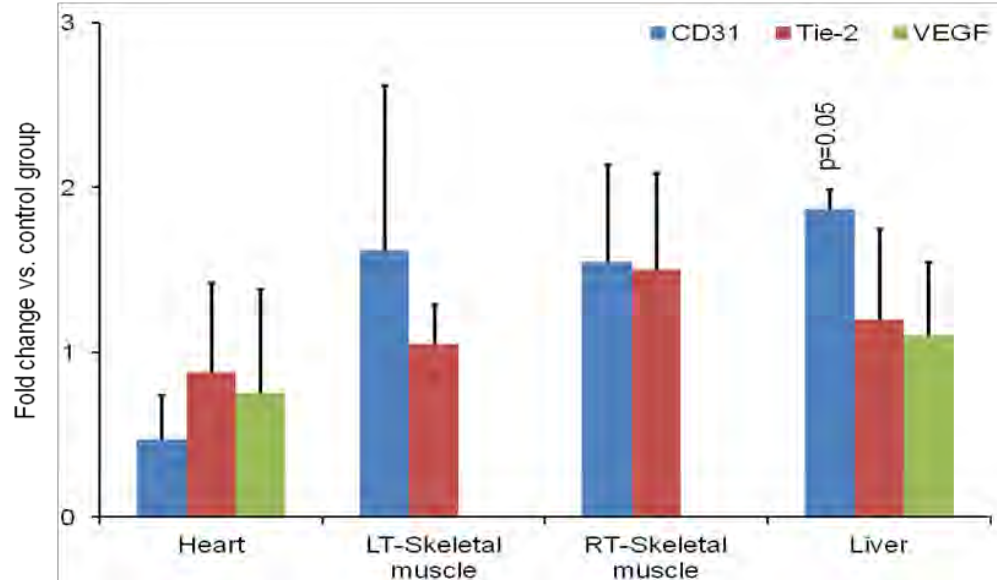


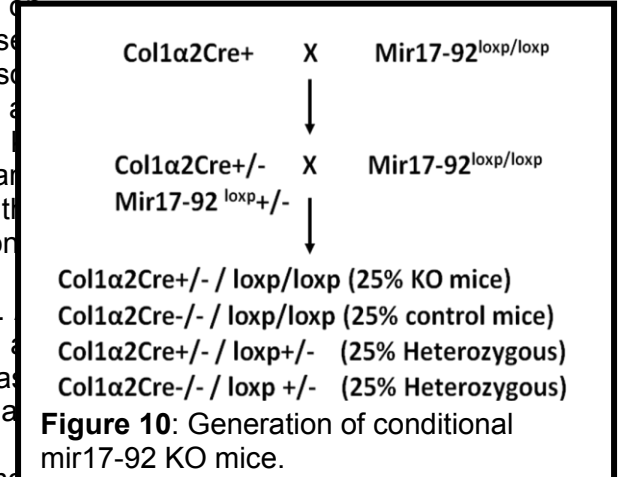
Figure 9: Expression levels of vascular genes (CD31, Tie-2 and VEGF) in mice treated with antagonist against mir-92 vs. mice treated with control antagonist. The y-axis corresponds to fold change and x-axis represents different tissues. Values are mean \pm SD. N=4.

Hot Start Taq polymerase] [Applied Biosystems, Foster City, CA], 50nM of primers, 15µl of water, and 5µl of template. The thermal conditions consisted of an initial denaturation at 95°C for 10 minutes followed by 40 cycles of denaturation at 95°C for 15 seconds (sec), annealing and extension at 60°C for 1 minute, and a final step melting curve of 95°C for 15 sec, 60°C for 15 sec, and 95°C for 15 sec. The data were analyzed using SDS software, version 2.0, and the results were exported to Microsoft Excel for further analysis. Data normalization was accomplished using the endogenous control [β -actin] and the normalized values were subjected to a $2^{-\Delta\Delta Ct}$ formula to calculate the fold change between the antagomir and control antagomir treated mice.

Surprisingly, we failed to detect any significant changes in the expression levels of vascular genes (VEGF, CD31 and Tie2) in heart, liver or skeletal muscle at the time points examined (**Figure 9**). We offer the following potential explanations for the negative results in this study: 1) the dosage of antagomir used in our study was slightly less compared to earlier study (2.7 mg/kg of body weight vs. 8 mg/kg of body weight) (2). The issue of whether higher dosage is required to see a change in the phenotype or whether the inhibitory effect on angiogenesis seen in the previous study was due to secondary effects on other genes require further evaluation. 2) The expression levels of angiogenic genes were examined only at one time point and additional time points may need to be examined. 3) Earlier study measured an increase in angiogenesis by blood flow while we examined expression levels of vascular genes.

Based on our above findings, we conclude that systemic administration of antagomir against miR-92, while it reduced expression levels of miR-92 in the skeletal muscle, liver and heart; it did not significantly alter either angiogenic or osteogenic response, thus suggesting possible redundancy in miR-92 regulation of angiogenesis.

Since there is a discrepancy between our findings and the published report, we focused on generating bone specific microRNA knockout mice to test the roles of microRNAs involved in regulating angiogenesis and osteogenesis in relation to exercise. We focused on miR17-92 cluster because recent in vitro findings revealed that miR92 is critical for osteoblast differentiation (16). Furthermore during the first year of funding, we found miR20a, belonging to the family of miR17-92 cluster is increased in response to loading. To date though there is no direct evidence about the role of miR in relation to bone, there is no clear in vivo evidence. Based on these rationale, we focused on generating miR17-92 cluster bone specific KO. We purchased homozygous miR17-92^{lox/lox} females (obtained from Jackson laboratory) crossed with transgenic male expressing an improved Cre recombinase under the control of a 100 bp promoter/enhancer unit of Col1a2 gene to generate Cre+ and Cre- miR17-92 loxP heterozygous mice. We crossed the heterozygous back with lox/lox parent to generate conditional mir17-92 knockout (cKO) mice and littermate controls. A schematic representation of breeding is shown in **Figure 10**. At 3 weeks, tail snips were collected and DNA was isolated as described earlier. Using genotype specific primer, polymerase chain reaction was performed on the pups to segregate conditional knockout mice from littermate controls.



Initial screening by DEXA analysis revealed that female mice deficient for osteoblast derived miR17-92 cluster **showed 24-28% and 18-21% reduction in BMC and area at 4 and 8 weeks of age (Table 5)**. Similarly, **male mice also showed 20% and 18% reduction in BMC and area at 4 and 8 weeks (Table 5)**. Overall DEXA analysis show that loss miR17-92 cluster affected skeletal growth in both sexes. Since female mice are less aggressive and amicable to grouping than male, we focused on female mice for further study.

To further validate the DEXA data, we measured skeletal parameters using pQCT or micro-CT in a 12 week old female cKO and WT mice. As can be seen in the micro-CT image, the miR17-92 cKO mice showed reduced cross sectional area when compared to WT mice (**Figure 11**). Furthermore quantitative analysis by micro-CT revealed that the reduced BMC at 4 and 8 weeks was maintained at 12 weeks and was caused by reduction in bone length (10% P<0.05) and cross sectional area (CSA, 18% P<0.05) but not by a vBMD change as measured by pQCT and micro-CT. These data illustrate that miR17-92 cluster is critical for osteoblast function in particular for promoting longitudinal and cross sectional skeletal growth.

In our preliminary study, we found that ML by four-point bending caused 2 fold increases in miR20a expression and since mice deficient for osteoblast derived miR17-92 cluster showed reduced cross sectional area (CSA) we predicted that miR17-92 cluster is necessary for normal cortical bone response to loading. To test this, we choose ML by four-point bending method which induces periosteal bone formation. Since the size of bone (CSA) in the miR17-92 cKO mice was smaller compared to wild type, we predict that mice with smaller circumference tend to receive higher mechanical strain than mice with larger circumference. In order to assure that the difference in the skeletal anabolic response induced by ML is purely due to lack of miR17-92 cluster rather than due to difference in strain, we need loads that produce same mechanical strain on both sets of mice. Using the **mathematical formula** previously described (17), the strain data was calculated and loads were adjusted to produce similar **mechanical strain ($4558\mu\epsilon \pm 355$ at 7N for KO mice vs. $4555\mu\epsilon \pm 454$ at 9N for WT mice, N=5)** in tibia of both mice. In our experience, that the calculated strain is generally higher than the measured strain.

Therefore, the amount of strain produced by respectively load, mentioned above is approximate.

Based on these data, we applied 7N load to miR17-92 cKO mice and 9N load to WT mice. The loading was performed for 2

weeks (6 days/week, 1 day rest) at 2Hz frequency for 36 cycles, once per day. pQCT analysis revealed that **2 weeks ML caused 28%, 38% and 13% increase in CSA, BMC and total vBMD respectively in the WT mice. In contrast, in the miR17-92 cKO mice, the changes were 8%, 15% and 9% in the above parameters response to ML.** Though, the miR17-92 cKO mice showed increase in bone response to loading, the magnitude increase was less when compared to WT mice ($P < 0.05$) providing first in vivo evidence that osteoblast produced miR17-92 cluster is important for normal periosteal bone response to loading.

(A) Female mice

Parameters	WT		miR17-92 cluster KO	
	4 wks	8 wks	4 wks	8 wks
BMD g/cm^2	$0.03 \pm 0.000^*$	$0.04 \pm 0.002^*$	0.03 ± 0.001	0.04 ± 0.002
BMC g	$0.17 \pm 0.02^*$	$0.37 \pm 0.03^*$	0.13 ± 0.009	0.29 ± 0.015
Area cm^2	$5.25 \pm 0.53^*$	$8.44 \pm 0.49^*$	4.32 ± 0.32	7.09 ± 0.08

(B) Male mice

Parameters	WT		miR17-92 cluster KO	
	4 wks	8 wks	4 wks	8 wks
BMD	0.03 ± 0.001	0.04 ± 0.00	0.03 ± 0.001	0.04 ± 0.001
BMC	0.16 ± 0.01	0.36 ± 0.025	0.13 ± 0.02	0.3 ± 0.04
Area	5.28 ± 0.52	8.44 ± 0.67	4.45 ± 0.37	7.22 ± 0.89

Table 5: DEXA measurement of skeletal parameters between miR17-92 KO and WT mice. Values are mean \pm SD, * $P < 0.05$ vs. miR17-92 cluster KO mice, (N=4-6).

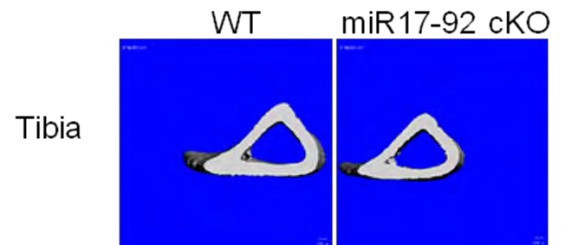


Figure 11: Cross sectional area of the tibia (3 mm away from the tibia-fibular junction) from WT and miR17-92 cKO mice.

Key Research Accomplishments

From task 1

1. Short duration of loading was not sufficient to stimulate osteogenic, angiogenic and miR expression.
2. We have chosen four-point bending method of loading than axial method to test the proposed aims.
3. The magnitude of angiogenic and osteogenic response induced by mechanical loading was higher in the four-point bending method of loading than axial method of loading.

4. We have identified miR93, miR-20a, miR16 and miR146a are differentially expressed in response to mechanical loading while miR15b did not show any significant change in response to loading. The issue of whether these MicroRNAs have a positive or negative effect on bone formation in response to loading is yet to be studied.
5. We have also identified miR-221 and 222 were not found to be expressed in bone.
6. Mice deficient for osteoblast derived miR17-92 cluster showed reduced longitudinal and cross sectional skeletal growth.
7. We have shown that miR93, miR-20a, miR16 and miR146a showed positive correlation and miR15b showed negative correlation with angiogenic marker genes.
8. Subsequently, we have also shown that miR-20a, miR16 and miR146a showed positive correlation with both mechanical loading (four-point bending method) induced increase in angiogenic and osteogenic marker genes.

From task 2

1. Antagomir injection (Mir92 or control antagomir) in mice did not cause any gross pathology.
2. Blocking miR92 did not affect osteogenesis (as evident from micro-CT analysis) induced by mechanical loading.
3. Blocking miR92 using corresponding antagomir affected miR92 expression but not angiogenic marker genes in heart, liver and skeletal muscle of experimental mice when compared to control mice treated with control-antagomir.

Additional Key Research Accomplishments

1. Generated mice deficient for osteoblast derived miR17-92 cluster.
2. Mice deficient for osteoblast derived miR17-92 cluster showed reduced longitudinal and cross sectional skeletal growth.
3. Magnitude of bone anabolic response induced by exercise is affected in mice with conditional disruption of osteoblast derived miR17-92 cluster when compared to the controls.

Reportable outcomes

1. Antony S, Mohan S, Wergedal JE, Rungaroon J and **Kesavan C**. Systemic Administration of an Antagomir Designed to Inhibit miR-92, a Regulator of Angiogenesis, Failed to Modulate Skeletal Anabolic Response to Mechanical Loading. 34th ASBMR Conference.
2. Sengul A, Santisuk R, Xing W, **Kesavan C**. Systemic administration of an antagomir designed to inhibit miR-92, a regulator of angiogenesis, failed to modulate skeletal anabolic response to mechanical loading. *Physiol Res*. 2013 Apr16; 62(2):221-6.
3. **Kesavan C**, Wergedal and Mohan S. Conditional Disruption of miR17-92 cluster in Osteoblasts Impairs Skeletal Growth and Periosteal Response to Mechanical loading. **Accepted for Oral presentation**. Annual Meeting of the American Society of Bone and Mineral Research, Baltimore, USA, 2013.

Conclusion

Systemic administration of antagomir against miR-92 while reduced expression levels of miR-92 in the tissues; it did not significantly alter either angiogenic or osteogenic response, thus suggesting possible redundancy in miR-92 regulation of angiogenesis.

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Appendices.....

List of personnel receiving pay from this research effort:
 Chandresekhar Kesavan, Joe Rungaroon, Anil Kapoor

PROJECTED and COMPLETED STATEMENT OF WORK

Task 1: Evaluate expression levels of Micro-RNA in response to mechanical loading (**Page 2-7, Completed**).

Sub-Task

1. Purchase 48 female (n=12/group) C5BL/6J mice from Jackson laboratory (**Page 1-2, Completed**).
2. Perform mechanical loading on these mice using tibia axial loading model (**Completed**).
Note: we have performed ML using four-point bending (**Page 2-3, Figure 1-3**) and axial method of loading (**Page 4-5, Figure 4-5**) to select the loading model that shows maximum biological change.
3. Euthanize the mice at different time points after the last loading (6hrs, 24 hrs, 3- and 7-days) and collect tissue samples for further analysis (**Page 2, Completed**).
4. Perform RNA extraction, RNA quality and RNA quantity (**Completed**).
5. Quantitate expression levels of angiogenesis specific MicroRNAs, angiogenesis and bone formation markers in the loaded and non-externally loaded bones using Real Time RT-PCR approach (**Completed; In page 3, see Figure 3 and table-1; In page 5, see Figure 5 and table-2 and In page 6, see Figure 6**).
6. Prioritize MicroRNAs for functional testing based on magnitude of loading induced changes in expression and what is known in the literature on the formation of MicroRNAs (**Completed, page 7**).

Projected Deliverables

1. We will establish experimental proof of evidence that MicroRNA levels are changed in response to mechanical loading in bone.
2. We will identify MicroRNAs that are involved in bone response to mechanical loading by identifying MicroRNAs that are known to control angiogenesis.

Accomplished outcome

1. We have provided evidence that short duration of loading was not sufficient to stimulate osteogenic, angiogenic and miR expression (**Completed, see page 1-2**).
2. We have chosen four-point bending method of loading than axial method to test the proposed aims (**Completed, see page 5-6**).
3. We have identified miR93, miR-20a, miR16 and miR146a are differentially expressed in response to mechanical loading while miR15b did not show any significant change in response to loading (**Completed, see page 6 and Figure 6**).
4. We have also identified miR-221 and 222 were not found to be expressed in bone (**Completed, see page 6 and Figure 6**).
5. We have shown that miR93, miR-20a, miR16 and miR146a showed positive correlation and miR15b showed negative correlation with angiogenic marker genes (**Completed, see page 6 and Table-3**).
6. Subsequently, we have also shown that miR-20a, miR16 and miR146a showed positive correlation with both mechanical loading (four-point bending method) induced increase in angiogenic and osteogenic marker genes (**Completed, see page 6 and Table-3**).

Task 2: Determine angiogenesis and bone anabolic response to mechanical loading by blocking identified MicroRNA using antisense oligos complementary to the specific MicroRNA.

Sub-Task

1. Purchase 24 female C57BL/6J mice from Jackson Laboratory (**Completed, see page 7**).
2. Purchase antisense oligomers to selected MicroRNAs (**Completed, see page 7**).
3. Test, if the antisense to selected MicroRNAs blocks angiogenesis *in vitro* (**Not completed; This is because a recent study using in vitro approach have demonstrated that blocking miR92 using antagomir increases angiogenesis**).
4. Based on this *in vitro* data and specific aim-1 results, we will inject mice with antisense oligo RNA complementary to specific MicroRNA that control angiogenesis. The route of injection will be intravenously and the dosage will be 8 mg per kg of body weight as described earlier (Science **324**(5935):1710-3) (**Completed, see page 8**).
5. Subject these mice and control group injected with scrambled antisense oligomer for 2 weeks of axial loading (**Completed, see page 8**).
6. Compare skeletal changes and bone strength between experimental and control mice by using *in vivo* Micro-CT (**Completed, see page 8**).
7. Euthanize the mice and collect tissues samples (**Completed, see page 9**).
8. Evaluate cellular processes contributing to osteogenesis and angiogenesis between experimental and control mice by performing histomorphometric analysis (**Not completed because there was no difference between control and experimental animals**).

Deliverables

1. We will establish experimental proof of experimental evidence that MicroRNA levels affect angiogenesis, bone formation in response to mechanical loading.
2. We will write up our results from task 1 and 2 for a publication in a high impact journal as well as present our data at relevant research meetings.

Accomplished outcome

4. Antagomir injection (Mir92 or control antagomir) in mice did not cause any gross pathology (**Completed, in page 7, see Table-4**). .
5. Blocking miR92 did not affect osteogenesis (as evident from micro-CT analysis) induced by mechanical loading (**Completed, in page 8, see Figure 7**).
6. Blocking miR92 using corresponding antagomir affected miR92 expression but not angiogenic marker genes in heart, liver and skeletal muscle of experimental mice when compared to control mice treated with control-antagomir (**Completed, in page 8 see figure 8 and in page 9, see figure 9**).